Please note: the shared protocols described herein may not have been validated by Pacific Biosciences and are provided as-is and without any warranty. Use of these protocols is offered to those customers who understand and accept the associated terms and conditions and wish to take advantage of their potential to help prepare samples for analysis using the PacBio® System. If any of these protocols are to be used in a production environment, it is the responsibility of the end user to perform the required validation.

20-kb Template Preparation Using BluePippin™ Size-Selection System (15-kb Size Cutoff)

Before You Begin

This procedure can be used to prepare size-selected libraries from 5 µg of sheared DNA using a 15-kb cutoff in the BluePippin Size-Selection system. With the "**0.75%, DF Marker S1 high-pass 15 kb – 20 kb**" protocol, size-selection cutoffs can be set to 15 kb up to 20 kb. This defines the lower edge of the range to be collected. Because size selection is aggressive, library yield using a 15-kb cutoff may be heavily impacted depending on the distribution of the starting sheared DNA. Therefore, it is imperative to QC samples prior to shearing.

Running DNA on Pulsed-Field Gel Electrophoresis (PFGE), Field-Inversion Gel Electrophoresis (FIGE), or any other electrophoretic system that provides good separation and accurate fragment sizing is highly recommended. High molecular weight DNA migrates as a band at approximately 48 kb (Bio-Rad 8-48 kb DNA size standard) when run on PFGE or FIGE. See Sample 1 in Figure 1 as an example of genomic DNA with high molecular weight. Although the sample appears to be slightly fragmented (smeared), the majority of the DNA is high molecular weight as shown by a strong band at approximately 48 kb. With this DNA quality, proceed to the shearing step outlined in the "Fragment and Concentrate" section.

If the sample is severely fragmented as in the Sample 2, you may eliminate the shearing step and proceed to library construction directly. However, a 15-kb cutoff may not be appropriate for this sample since it could result in a loss of the majority of the sample. Consider using a less aggressive sizing cutoff (6 kb - 10 kb).

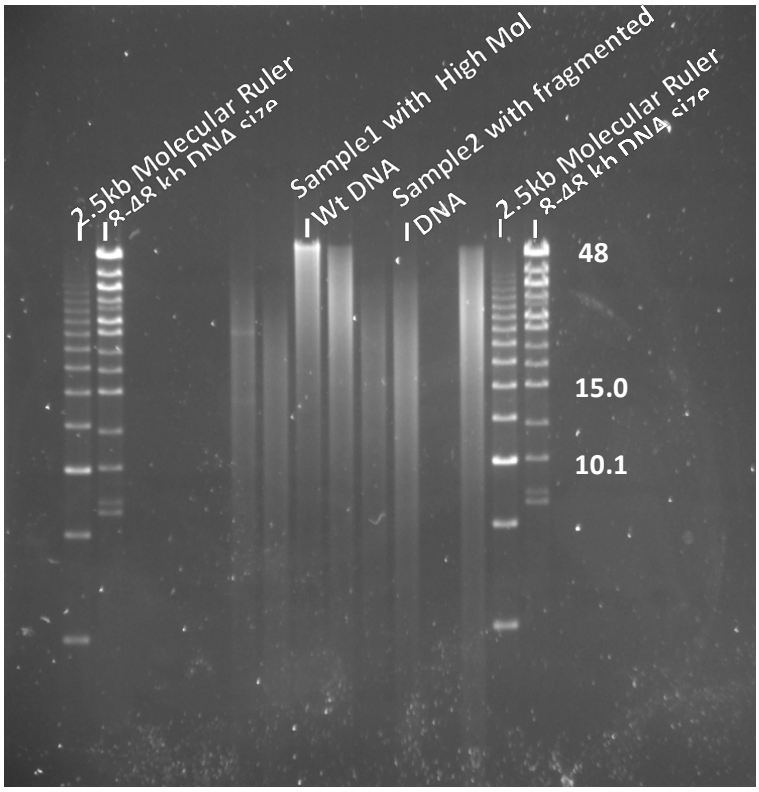

Figure 1: FIGE (Pippin Pulse) of 5-Mb genome. Examples of high molecular weight DNA (Sample 1) and fragmented DNA (Sample 2).

A good shearing strategy is also key to constructing a good 20-kb library using a 15-kb cutoff. Use the shearing procedure outlined in the "Fragment and Concentration" section. An example of sheared DNA using the recommended procedure is shown in Figure 2. Ideally, the mode of fragment distribution should be on or larger than the 17-kb marker. Perform tests to determine the best shearing condition for your sample. Over-shearing will result in very low yield.

With 15 kb as the size cutoff, 10 µg sheared gDNA going into the repair steps will typically generate sufficient size-selected libraries for large-genome sequencing projects. This procedure is optimized for 5 µg of sheared gDNA. If working with 10 µg of sheared gDNA, scale all the reaction volumes proportionally (e.g., if the input amount of DNA is double the amount set forth in this procedure, double all the reaction volumes listed in the tables).

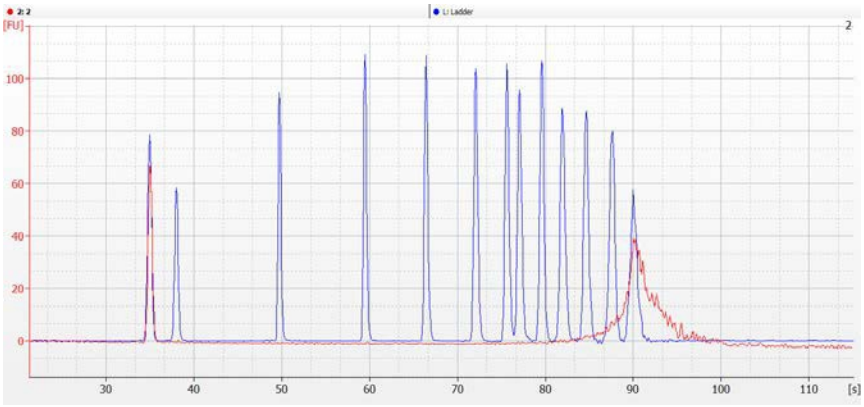

Figure 2: Bioanalyzer trace of a 20-kb *E. coli* sheared DNA. Ideally, fragment distribution should migrate on, or be larger than, the 17-kb marker (12000 ladder). If the mode is smaller than 17 kb, a majority of the sample may be lost when using a 15-kb size selection cutoff.

If using this protocol for the first time, we strongly recommend that you process a control sample first. Using the DNA shearing methods and subsequent AMPure PB bead purification steps described below, you should recover approximately **50%-80%** of your input DNA (by mass). Typical yields, from pre-purified DNA (where smaller fragments are already eliminated as a result of the shearing process) are between 80-100%.

Insert Size Target	Insert Size Range	Sheared and Concentrated DNA Amount	Ligation	
20 kb	15 kb to 20 kb (size-selected using BluePippin system)	5 µg	Blunt	

DNA Handling:

When constructing large insert libraries, we highly recommend using gentle mixing instead of vortexing. Vigorous vortexing can potentially damage large fragments. Gentle mixing can be done on a rotator (same rotator used for MagBead binding) at room temperature for 20 minutes.

BluePippin™ System Recommendations:

Refer to the table of recommendations below for guidelines when using the BluePippin system. Yield, after the size-selection step, depends on shear distribution.

Mass of SMRTbell Library	Size Selection Cut-off Requirement	Recommended Cutoff (bp)		Cassette Definition File	Ver-sion	Mar-ker	Kit Part Number
		BP start	BP end*				
< 2 µg	4,000 to 5,000 kb	4,000	50,000	0.75%DF Marker S1 high-pass 4-10kb v2	v2	S1	PAC20KB or BLF7510
		5,000	50,000	0.75%DF Marker S1 high-pass 4-10kb v2	v2	S1	PAC20KB or BLF7510
>600 ng	6,000 kb to 10,000 kb	6,000	50,000	0.75%DF Marker S1 High-Pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		7,000	50,000	0.75%DF Marker S1 High-Pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		8,000	50,000	0.75%DF Marker S1 High-Pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		9,000	50,000	0.75%DF Marker S1 High-Pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
		10,000	50,000	0.75%DF Marker S1 High-Pass 6-10kb v3	v3	S1	PAC20KB or BLF7510
>5 µg	15,000 kb to 20,000 kb	15,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		16,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		17,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		18,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		19,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510
		20,000	50,000	0.75%DF Marker S1 high-pass 15-20kb	0	S1	PAC20KB or BLF7510

*BP end should always be set to 50,000

Fragment and Concentrate DNA

Gentle mixing is recommended for large-insert libraries (20 kb), 15-kb size selected. Typical yields after shearing and AMPure purification are 50-70%, depending on the quality and purity of the input gDNA.

Use a Covaris® g-TUBE® device to shear > 10 µg DNA sample. The most up-to-date guidance on how to use the g-TUBE device, along with recommended centrifuges and centrifugation speeds, can be found in the g-TUBE device user manual available for download from the Covaris website or the Shared Protocols page of SampleNet, with the recommendations below. It is highly recommended to perform test shears first to ensure fragment distribution is on or above the 17-kb marker of the Bioanalyzer instrument.

1. Dilute your DNA concentration to 100-300 ng/µL in Elution Buffer (EB). PacBio recommends a sample volume of at least 100 µL.
2. Shear at 4800 rpm for 2 minutes in an Eppendorf® MiniSpin plus.
3. Check for residual volume remaining in the upper chamber. If present, spin again at 4800 rpm for an additional two minutes. Repeat this spin cycle until most if not all of the sample has passed through the orifice.
4. If a small volume persists, perform a final spin at 8000 - 10000 rpm to push everything through the orifice. Do not do this step if a large volume of your sample is still present in the upper chamber. You may also use a pipette to remove the residual volume that does not make it through the orifice.
4. Invert and spin at 4800 until all samples have passed through the orifice.
5. Recover your sample into a 1.5 or 2.0 mL LoBind microcentrifuge tube. Add EB if necessary to adjust volume to 100 µL for the concentration step below.

STEP	✓	Concentrate DNA	Notes
1		Add **0.45X** volume of AMPure® PB magnetic beads. _____µL of sample X **0.45X** = _____µL of beads Note that the beads must be brought to room temperature and all AMPure PB bead purification steps should be performed at room temperature. Before using, mix the bead reagent well until the solution appears homogenous. Pipette the reagent slowly since the bead mixture is viscous and precise volumes are critical to the purification process.	
2		Mix bead/DNA solution thoroughly by tapping the tube gently. Do not pipet to mix.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle end-over-end rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack until the beads collect to the side of the tube and the solution appears clear. The actual time required to collect the beads to the side depends on the volume of beads added.	
7		With the tube still on the magnetic bead rack, slowly pipette off cleared supernatant and save in another tube. Avoid disturbing the bead pellet. If the DNA is not recovered at the end of this Procedure, you can add equal volumes of AMPure PB beads to the saved supernatant and repeat the AMPure PB bead purification steps to recover the DNA.	
8		Wash beads with freshly prepared 70% ethanol. Note that 70% ethanol is hygroscopic and should be prepared FRESH to achieve optimal results. Also, 70% ethanol should be stored in a tightly capped polypropylene tube for no more than 3 days. – Do not remove the tube from the magnetic rack. – Use a sufficient volume of 70% ethanol to fill the tube (1.5 mL for 1.5 mL tube or 2 mL for 2 mL tube). Slowly dispense the 70% ethanol against the side of the tube opposite the beads. Let the tube sit for 30 seconds. – Do not disturb the bead pellet. – After 30 seconds, pipette and discard the 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	

STEP	✓	Concentrate DNA	Notes
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with the tube caps open) for 30 to 60 seconds.	
13		Calculate appropriate volume of Elution Buffer. _____ng X **0.5** / (_____ng/µL) = _____µL of Elution Buffer needed The minimum DNA concentration required to proceed to the next step (End-Repair) is **140 ng/µL** with preferred mass of at least **5 µg**.	
14		Add the Pacific Biosciences® Elution Buffer volume (calculated in step 13) to your beads. Close the tube and tap with finger to mix. Do not pipet to mix. – Elute the DNA by gentle rotation/mixing for 20 minutes at room temperature. – Spin the tube down to pellet beads, then place the tube back on the magnetic bead rack. – Perform concentration measurements. Verify your DNA concentration using a Nanodrop® or Qubit® quantitation platform. If the DNA concentration is estimated to be equal to or below **12 ng/µL**, a Qubit system reading is required. When performing a Qubit system reading, ensure that your sample is within the range of the Qubit kit you are using. For proper concentration calculations, incorporate the dilution factor (used when diluting your sample) to be within range of the Qubit kit and the dilution factor when diluting your sample with the working solution. The latter part of this dilution factor can be calculated automatically by the Qubit system. – Discard the beads.	
15		Perform qualitative and quantitative analysis using a Bioanalyzer® instrument. Note that the Bioanalyzer instrument has different kits in its offering and the appropriate kit, based on insert size, should be used. Dilute the samples appropriately before loading on the Bioanalyzer chip so that the DNA concentration loaded falls well within the detectable minimum and maximum range of the assay. Refer to Agilent Technologies' guides for specific information on the range of the specific kit you might be using. Note that typical yield, at this point of the process (i.e. post-shearing and after one **0.45X** AMPure PB bead purification), is approximately **50%-80%**.	
16		The sheared DNA can be stored for up to 24 hours at 4°C or at -20°C for longer duration.	
17		Actual recovery per µL and total available sample material: _____	

ExoVII Treatment of DNA

Use the following table to remove single-stranded ends from DNA fragments. If preparing larger amounts of DNA, scale the reaction volumes accordingly (i.e., for 10 µg of DNA scale the total volume to 100 µL). Do not exceed 100 ng/µL of DNA in the final reaction.

1. In a LoBind microcentrifuge tube, add the following reagents:

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
Sheared DNA	–		__ µL for 5.0 µg	–		
DNA Damage Repair Buffer		10 X	5.0 µL	1 X		
NAD+		100 X	0.5 µL	1 X		
ATP high		10 mM	5.0 µL	1 mM		
dNTP		10 mM	0.5 µL	0.1 mM		
ExoVII		10 U/µL	1.0 µL	0.2 U/µL		
H_2O	–		__ µL to adjust to 50.0 µL	–		
Total Volume			50.0 µL	–		

2. Mix the reaction well by gently tapping the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 37°C for 15 minutes, then return the reaction to 4°C.

Repair DNA Damage

Use the following table to prepare your reaction.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (ExoVII treated)	–		50 µL	–		
DNA Damage Repair Mix		25 X	2.0 µL	1X		
Total Volume			52.0 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 37°C for 20 minutes, return the reaction to 4°C for 1 to 5 minutes.

Repair Ends

Use the following table to prepare your reaction, then purify the DNA.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (Damage Repaired)	–		52.0 µL	–		
End Repair Mix		20 X	2.5 µL	1X		
Total Volume			54.5 µL	–		

1. Mix the reaction well by gently tapping the tube.
2. Spin down contents of tube with a quick spin in a microfuge.
3. Incubate at 25°C for 5 minutes, return the reaction to 4°C.

STEP	✓	Purify DNA	Notes
1		Add **0.45X** volume of AMPure PB beads to the End-Repair reaction. (For detailed instructions on AMPure PB bead purification, see the Concentrate DNA section.)	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **20 µL** Elution Buffer. Mix by gently tapping the tube, elute by rotating the tube for 20 minutes at room temperature.	
14		Optional: Verify your DNA amount and concentration using a Nanodrop or Qubit quantitation platform, as appropriate.	
15		Optional: Perform qualitative and quantitative analysis using a Bioanalyzer instrument with the DNA 12000 Kit. Note that typical yield at this point of the process (following End-Repair and one **0.45X** AMPure PB bead purification) is approximately between **80-100%** of the total starting material.	
16		The End-Repaired DNA can be stored overnight at 4°C or at -20°C for longer durations.	
17		Actual recovery per µL and total available sample material: _____	

Prepare Blunt-Ligation Reaction

Use the following table to prepare your blunt-ligation reaction:

1. In a LoBind microcentrifuge tube (on ice), add the following reagents in the order shown. If preparing a Master Mix, ensure that the adapter is NOT mixed with the ligase prior to introduction of the inserts.

Reagent	Tube Cap Color	Stock Conc.	Volume	Final Conc.	✓	Notes
DNA (End Repaired)	–		19.0 µL to 20.0 µL			
Annealed Blunt Adapter (20 µM)		20 µM	10* µL	5 µM		
Mix before proceeding						
Template Prep Buffer		10 X	4.0 µL	1X		
ATP low		1 mM	2.0 µL	0.05 mM		
Mix before proceeding						
Ligase		30 U/µL	1.0 µL	0.75 U/µL		
H_2O	–	–	__ µL to adjust to 40.0 µL	–		
Total Volume	–	–	40.0 µL	–		

*Note that this increase in adapter during ligation minimizes the incidence of chimeras. Adapter dimers are then efficiently removed during size selection in the BluePippin System. This is not recommended for libraries which are not being size selected using the BluePippin System.

2. Mix the reaction well by gently tapping the tube.
3. Spin down contents of tube with a quick spin in a microfuge.
4. Incubate at 25°C overnight.
5. Incubate at 65°C for 10 minutes to inactivate the ligase, then return the reaction to 4°C. You must proceed with adding exonuclease after this step.

Add exonuclease to remove failed ligation products.

Reagent	Tube Cap Color	Stock Conc.	✓	Volume
Ligated DNA				40 µL
Mix reaction well by pipetting				
ExoIII		100.0 U/µL		1.0 µL
ExoVII		10.0 U/µL		1.0 µL
Total Volume				42 µL

1. Spin down contents of tube with a quick spin in a microfuge.
2. Incubate at 37°C for 1 hour, then return the reaction to 4°C. You must proceed with purification after this step.

Purify SMRTbell™ Templates

STEP	✓	Purify SMRTbell Templates	Notes
1		Add **0.45X** volume of AMPure PB beads to the exonuclease-treated reaction. (For detailed instructions on AMPure PB bead purification, see the Concentrate DNA section).	
2		Mix the bead/DNA solution by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads. Do not pellet beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin to pellet beads. Both the beads and any residual 70% ethanol will be at the bottom of the tube. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute the DNA off the beads in **31 µL** of Elution Buffer. Mix by gently tapping the tube, elute by rotating the tube for 20 minutes at room temperature	

BluePippin™ Size Selection

Follow the recommendations below and the BluePippin User Manual and Quick Guide (to www.sage-science.com) to size-select your ~20-kb SMRTbell templates using the BluePippin instructions. Be sure you have reviewed the recommendations listed in the *User Bulletin - Guidelines for Preparing 20 kb SMRTbell™ Templates.*

Note that you must use the BluePippin "0.75%, DF Marker S1 high-pass 15 kb – 20 kb" protocol for this procedure. It is highly recommended to upgrade the BluePippin Software to **v6.20** to enable simultaneous sample elution and the auto-lights off feature. If you have upgraded to v6.20, skip this section and proceed to "Prepare DNA Samples for Each Lane". See the table below for features of each software version.

Software Version	Elution	Blue LED Lights
< 6.11	One sample at a time	Turn off manually
6.11	Simultaneous elution	Turn off manually
6.20	Simultaneous elution	Auto lights off

If you have not upgraded to v6.20 and are running v6.11 or lower, follow the instructions below on how to "Calibrate and Turn Off LEDs". During the elution step, samples may be exposed to blue LEDs as they are queued for elution. This exposure may result in damage to the SMRTbell templates. We recommend turning off the blue LEDs for all lanes except the S1 Marker lane.

If you are running v6.11 or lower, it is recommended to run the BluePippin system overnight when running several samples. Separation time is approximately 3 hours and elution time is approximately 45 minutes.

STEP	✓	Calibrate and Turn Off LEDS	Notes
1		Place the calibration fixture on the nest of the instrument and close the lid. Click "Calibrate" from the main screen. Click "Calibrate" in the calibration pop up window.	
2		After calibration passes, click "Exit".	
3		From the main screen, click on the BluePippin logo located in the lower right corner.	
4		Enter the password "pips" to display advanced user tabs.	
5		Select the "LED Setup Tab".	
6		Adjust the LED Counts to zero in the lanes that you want to turn off the LED. Double click on the LED count to highlight the number and enter "0". IMPORTANT: Do not change the LED count in the lane that will contain the S1 reference marker.	
7		Click on "Apply (Enter)" to save calibration values.	
8		Verify that the LEDs are turned off.	
9		The instrument is now ready for use. Note that instrument calibration (step 1) will turn on all LEDs.	

For other applications that do not require LEDs to be turned off, set the instrument to hide the "advanced tabs":

1. Click on the BluePippin logo in the lower right corner of the main screen.
2. Do not enter a password in the pop up window.
3. Click "OK".

STEP	✓	Prepare DNA Samples for Each Lane	Notes
1		If necessary, dilute up to 5 µg SMRTbell templates into a final volume of 30 µL Elution Buffer. Run 500 ng to 5 µg SMRTbell templates per lane. It's not recommended to start with less than 500 ng per lane.	
2		Bring the Loading Solution to room temperature, then add 10 µL of the Loading Solution to the 30 µL DNA sample. The Loading Solution is viscous so pipet slowly to ensure it is completely transferred into the DNA sample. Mix by gentle mixing; do not vortex. Spin briefly to collect the contents at the bottom.	
3		Follow the manufacturer's recommendations to set up a run protocol. When setting up the run protocol, select the "0.75% DF marker S1 high-pass 15-20kb" cassette definition file. Choose the "Range" selection mode, and enter the desired "BPstart" value from 15000-20000 bp. Enter a "BP End" value of 50000 bp.	
4		Start the run.	
5		Collect eluate into a 1.5 or 2.0 mL LoBind microcentrifuge tube. Note that volumes may vary from approximately 40 to 60 µL. Proceed directly to AMPure purification at this point. Note: It is highly recommended to wait at least 45 minutes after the run terminates before removing the eluted DNA. This has shown an increase in recovery of SMRTbell libraries.	

Concentrate size-selected SMRTbell templates in a 1.5 or 2.0 mL LoBind microcentrifuge tube using 1X AMPure PB beads.

STEP	✓	Concentrate and Quantify Size-Selected Templates	Notes
1		Measure volume of eluate and overlay sample with an equal volume of AMPure PB beads.	
2		Mix the bead/DNA solution thoroughly by gently tapping the tube.	
3		Quickly spin down the tube (for 1 second) to collect the beads.	
4		Allow the DNA to bind to beads by gentle rotation for 20 minutes at room temperature. We recommend using a VWR tube rotator.	
5		Spin down the tube (for 1 second) to collect beads.	
6		Place the tube in a magnetic bead rack to collect the beads to the side of the tube.	
7		Slowly pipette off cleared supernatant and save (in another tube). Avoid disturbing the bead pellet.	
8		Wash beads with freshly prepared 70% ethanol.	
9		Repeat step 8.	
10		Remove residual 70% ethanol. – Remove tube from magnetic bead rack and spin quickly. – Place the tube back on magnetic bead rack. – Pipette off any remaining 70% ethanol.	
11		Check for any remaining droplets in the tube. If droplets are present, repeat step 10.	
12		Remove the tube from the magnetic bead rack and allow beads to air-dry (with tube caps open) for 30 to 60 seconds.	
13		Elute DNA from beads by adding 10 µL EB onto beads. Mix gently by tapping the tube. Elute the DNA by gentle mixing using a rotator for 20 minutes. NOTE: For up to 5 µg input DNA, elute in 10 µL. For > 5 µg input DNA (if multiple lanes were pooled for this step), scale the elution volume proportionately.	
14		Spin briefly to collect the contents at the bottom of the tube.	
15		Return sample to magnetic rack and let stand until beads are well-separated. Collect to the side of the tube.	
16		Transfer supernatant containing size-selected SMRTbell templates to a new LoBind microcentrifuge tube.	

STEP	✓	Concentrate and Quantify Size-Selected Templates	Notes
17		Use 1 µL of purified SMRTbell templates to make a 1:5 dilution in EB, and measure the DNA concentration using a Qubit fluorometer. Retain the remaining 4 µL of diluted sample for QC by FIGE. NOTE: Typical yields of size-selected, ~20 kb SMRTbell library from 500 ng - 5 µg input material are 20-40%.	

Anneal and Bind BluePippin™ Size-Selected SMRTbell™ Templates

Use the Binding Calculator to anneal sequencing primer at 0.833 nM concentration and bind polymerase at 0.500 nM concentration. These are the default concentrations required for 20 kb libraries. Note that you must have the PacBio DNA/Polymerase Kit and use LoBind microcentrifuge tubes for this step.

Before adding the primer to the SMRTbell template, the primer must be heated to 80ºC followed by a rapid cool-down to 4ºC. The conditioned primer can then be added to the SMRTbell template in 1X Primer Buffer, followed by incubation at 20 ºC for 30 minutes.

For polymerase binding, incubation at 30ºC for 30 minutes is sufficient. Instructions for polymerase binding are provided by the calculator.

For more information about using the Binding Calculator, see the Pacific Biosciences *Template Preparation and Sequencing Guide* and *QRC - Annealing and Binding Recommendations.*

Prepare for MagBead Loading

Optimal loading of size-selected ~20 kb tem plates using P6 polym erase can be achieved using an on-plate concentration of ~0.100 nM. An initial loading test on-plate concentration of 0.100 nM is highly recommended.

For efficient binding to Magnetic Beads, bound complexes (at 0.500 nM concentration) must be diluted in the appropriate ratio of MagBead Binding Buffer and MagBead W ash Buffer. Follow the Binding Calculator instructions to dilute your sample for MagBead binding.

Control Complex Dilution

If you will be using the PacBio Control Complex, dilute the DNA Control Complex according to the volumes and instructions specified in the Calculator.

Sequence

To prepare for sequencing on the instrument, refer to the *RS Remote Online Help* system or *Pacific Biosciences Software Getting Started Guide* for more information. Follow the touchscreen UI to start your run. Note that you must have a DNA Sequencing Kit and SMRT® Cells for standard sequencing.

When sequencing size-selected ~20 kb SMRTbell templates prepared by this method, be sure to indicate the magnetic bead collection protocol, a 20,000 bp insert size, stage start, and 240 minute movies when setting up your run protocol in RS Remote.

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